THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 8, pp. 5203–5213, February 20, 2015

Prevalence and Gene Characteristics of Antibodies with Cofactor-induced HIV-1 Specificity*[S]

Received for publication, October 11, 2014, and in revised form, December 24, 2014 Published, JBC Papers in Press, January 7, 2015, DOI 10.1074/jbc.M114.618124

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Background: Normal immune repertoires have a fraction of cofactor-binding antibodies.
Results: Heme exposure results in acquisition of reactivity to gp120 HIV-1 in 24% of antibodies in a seronegative immune repertoire; these antibodies have less mutated variable regions.
Conclusion: Human immune repertoires contain high frequency of antibodies with cofactor-induced antigen specificities.
Significance: This work enhances understanding of molecular features of heme-sensitive Abs and their contribution to diversification of the immune repertoires.

The healthy immune repertoire contains a fraction of antibodies that bind to various biologically relevant cofactors, including heme. Interaction of heme with some antibodies results in induction of new antigen binding specificities and acquisition of binding polyreactivity. *In vivo*, extracellular heme is released as a result of hemolysis or tissue damage; hence the post-translational acquisition of novel antigen specificities might play an important role in the diversification of the immunoglobulin repertoire and host defense. Here, we demonstrate that seronegative immune repertoires contain antibodies that gain reactivity to HIV-1 gp120 upon exposure to heme. Furthermore, a panel of human recombinant antibodies was cloned from different B cell subpopulations, and the prevalence of antibodies with cofactor-induced specificity for gp120 was determined. Our data reveal that upon exposure to heme, ~24% of antibodies acquired binding specificity for divergent strains of HIV-1 gp120. Sequence analyses reveal that heme-sensitive antibodies do not differ in their repertoire of variable region genes and in most of the molecular features of their antigen-binding sites from antibodies that do not change their antigen binding specificity. However, antibodies with cofactor-induced gp120 specificity possess significantly lower numbers of somatic mutations in their variable region genes. This study contributes to the understanding of the significance of cofactor-binding antibodies in immunoglobulin repertoires and of the influence that the tissue microenvironment might have in shaping adaptive immune responses.

Immune repertoires of all healthy individuals contain antibodies (Abs)[2] that are able to interact with different low molecular weight compounds, including biologically relevant cofactors as riboflavin, FMN, FAD, heme (Fe(II) protoporphyrin IX), and ATP, as well as metal ions (1–7). The binding affinity of riboflavin, FAD, and FMN to Abs is higher than the binding affinity to known plasma transporters of heterocyclic compounds such as albumin (1, 2). A fraction of Abs in normal serum also bind with high affinity to several xenobiotic organic compounds such as dinitrophenyl and naphthalene derivatives, as well as azo compounds (8–12). The presence of cofactor-binding Abs in normal human immune repertoires has been recently used for specific targeting of cancer cells or viruses as well as for catalysis of redox reactions, thus demonstrating the possibility to develop innovative therapeutic strategies based on cofactor-binding Abs (13–17). However, the origin, molecular characteristics, and physiopathological significance of natural cofactor-binding Abs in human immune repertoires remain not well understood.

Cofactor-binding Abs have initially been proposed to serve solely as inert carriers of biologically relevant heterocyclic molecules in plasma (1, 2). More recent studies, however, revealed that the binding of certain cofactors, *i.e.* heme, has a considerable impact on the antigen binding specificity of Abs. Thus, it has been demonstrated that normal immune repertoires contain a fraction of Abs that can acquire novel antigen binding specificities upon exposure to heme (7, 18–21). These Abs belong to different immunoglobulin isotypes (IgG, IgA, and IgM), and their exposure to heme results in the appearance of novel binding specificities for both self-derived and pathogen-derived antigens (7, 19, 22). Moreover, it has been observed that heme endows some monoclonal Abs with the capability to rec-

* This work was supported by grants from INSERM, CNRS, and UPMC-Paris 6 and by grants from the Centre de Recherche des Cordeliers (Prix Jeunes Chercheurs 2008) and Agence Nationale de la Recherche (Grant ANR-13-JCV1-006-01).
[S] This article contains supplemental Table 1.
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[2] The abbreviations used are: Abs, antibodies; gp120, glycoprotein 120; pnC, pneumococcal C-polysaccharide; CDR, complementarity-determining region(s); IVIg, intravenous immunoglobulin(s).

ognize numerous unrelated antigens, *i.e.* they acquire antigen binding polyreactivity (7, 23). Previous studies have demonstrated that the post-translational acquisition of novel antigen binding specificities by cofactor binding correlates with an increased anti-inflammatory activity of IgG (24). Importantly, extracellular heme can be released in large quantities as a result of intravascular hemolysis or tissue damage in numerous disease conditions (25–27). This may result in interactions of heme with circulating cofactor-binding Abs and induction of novel antigen binding specificities *in vivo*.

Little is known, however, about the frequencies of Abs with cofactor-inducible antigen specificities in human immune repertoires and the molecular features of the variable regions that determine their sensitivity to heme. To address these questions, we used a repertoire of human recombinant Abs obtained upon cloning of variable regions, amplified by single-cell PCR technology from different B cell subpopulations, and fused to Fcγ1 constant chain. We demonstrated that after heme exposure, ~24% of Abs in the repertoire gain an ability to recognize different variants of a highly heterogeneous envelope glycoprotein, gp120 of HIV-1. Most of the gp120 reactive Abs were also polyreactive and bound to unrelated proteins. Further, we analyzed the gene characteristics of variable regions of monoclonal Abs that may explain their tendency to acquire novel antigen binding specificities upon contact with heme.

EXPERIMENTAL PROCEDURES

Proteins and Antibodies

Recombinant envelope glycoprotein 120 (gp120) from HIV-1 strains BaL (clade B), CN54 (clade C), 96ZM651 (clade C), and 93TH975 (clade A/E) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, at the National Institutes of Health. Recombinant gp120 from strains 92RW020 (clade A) and JRCSF (clade B) were purchased from Immune Technology Corp. (New York, NY).

Human transferrin, human hemoglobin, and porcine tubulin were obtained from Sigma-Aldrich. Human factor IX was obtained from LFB, (Les Ulis, France), and pneumococcal C-polysaccharide (pnC) was obtained from Statens Serum Institut, (Copenhagen, Denmark). Human immunodeficiency virus immune globulin (HIVIg) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, at the National Institutes of Health. Two commercial IVIg preparations, Intratect (Biotest AG, Dreieich, Germany) and Endobulin S/D (Baxter, Deerfield, IL), were used as a source of normal pooled human immunoglobulins G. The repertoire of human antibodies was described previously in Refs. 28 and 29. Briefly, the variable genes encoding the antibody heavy and light chains were amplified by single-cell PCR from synovial tissue of rheumatoid arthritis patients, cloned in PUC19 vector containing the genes encoding the constant Fc-γ1 or λ/κ regions, respectively, and expressed using HEK293 cells (28).

Reagents

All reagents used in the study were analytical grade quality. Stock solutions of hematin (ferriprotoporphyrin IX hydroxide) were prepared by dissolving hemin (Fluka, St. Louis, MO) to concentrations of 1 mM in 0.05 N solution of NaOH or 20 mM in 0.1 N solution of NaOH. The treatment of immunoglobulins was always performed with freshly prepared hematin, at dim light conditions.

ELISA

Reactivity of IgG Repertoire to gp120—Ninety-six-well polystyrene plates (Nunc MaxiSorp, Roskilde, Denmark) were coated with recombinant HIV-1 gp120 variants, 92RW020, BaL, JRCSF, or CN54, diluted to 5 μg/ml in PBS. After incubation for 3 h at 22 °C, the residual binding sites on plates were blocked by PBS containing 0.25% Tween 20 (Sigma-Aldrich). In the first experimental setting, IVIg (67 μM, 10 mg/ml) diluted in PBS were exposed to a fixed concentration of heme (50 μM). After a 30-min incubation on ice, native and heme-exposed immunoglobulins were diluted serially in PBS containing 0.05% Tween 20 (PBS-T) to final concentrations of 1000, 333, 111, 37, 12, 4, 1.3, 0.45, 0.15, and 0.05 μg/ml for IVIg and HIVIg and then incubated with gp120 BaL-coated plates for 2 h at 22 °C. In the second experimental setting, a fixed concentration of IVIg (20 μM, 3 mg/ml) was exposed for 30 min on ice to increasing concentrations of heme: 0, 0.5, 1, 2, 4, 8, 16, 32, and 64 μM, respectively. Heme-exposed immunoglobulins were diluted in PBS-T to 150 μg/ml and incubated for 2 h at 22 °C with plates coated with gp120 variants: 92RW020, BaL, JRCSF, or CN54. After incubation with antibodies, in both experimental variants, microtitration plates were washed extensively with PBS-T and incubated with a peroxidase-conjugated mouse anti-human IgG (clone JDC-10, Southern Biotech, Birmingham, AL) for 1 h at 22 °C. Immunoreactivity of IgG were revealed by measuring the absorbance at 492 nm after the addition of peroxidase substrate, *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) and stopping the reaction by the addition of 2 N HCl.

Reactivity of IgG Repertoire to Autoantigens and pnC—Ninety-six-well polystyrene plates (Nunc MaxiSorp) were coated with human transferrin, human factor IX, human hemoglobin (apo-form), and pnC diluted to 10 μg/ml in PBS as well as with porcine tubulin diluted to 5 μg/ml in PBS. After incubation for 3 h at 22 °C, the residual binding sites on plates were blocked by PBS containing 0.25% Tween 20. For the treatment with heme, all recombinant Abs were diluted to 50 μg/ml and exposed to 10 μM final concentration of hematin. As a positive control, IVIg at 10 μM (1.5 mg/ml) was treated with 10 μM of hematin. After 30 min of incubation on ice, native and heme-exposed monoclonal Abs were first diluted with TBS-T (0.1% Tween 20) to final IgG concentrations of 25 μg/ml and incubated for 2 h at 4 °C. Further, the Abs were diluted in PBS-T to 6.25 μg/ml and incubated for 2 h at 22 °C with plates coated with the studied proteins or pnC. Heme-exposed IVIg diluted to 50 μg/ml in PBS-T was incubated on each plate. The next steps of ELISA were identical as these described above.

The binding of native and heme-exposed Abs to particular antigen was expressed as a percentage of the binding of the heme-exposed IVIg. The monoclonal Abs that demonstrated binding intensity toward the given antigen above a threshold value (defined as the mean reactivity of all native Abs plus five standard deviations) upon heme exposure were considered as heme-sensitive.

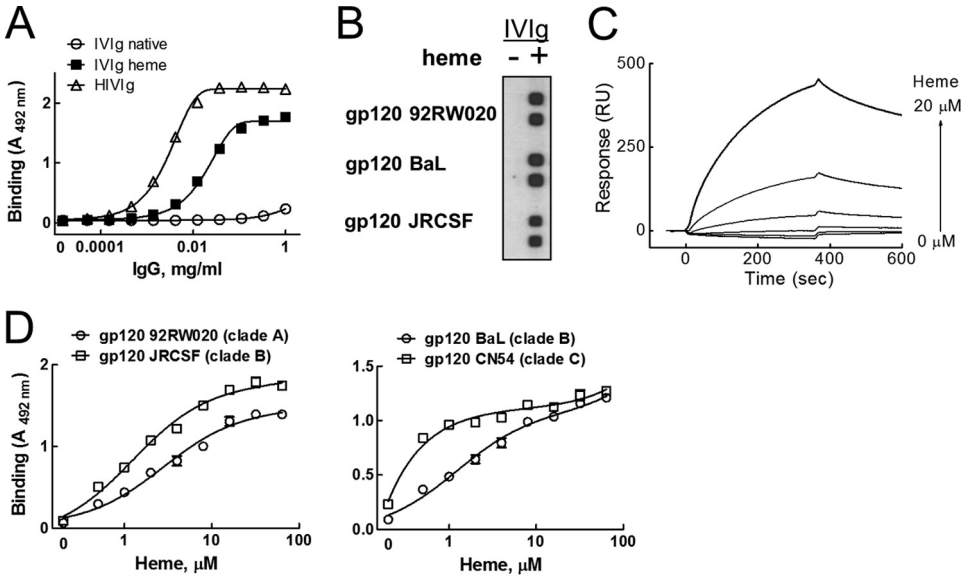

FIGURE 1. **Heme induces binding of pooled human IgG to gp120.** *A*, comparison of binding of native IVIg (*open circles*), heme-exposed IVIg (67 μM IgG/50 μM hematin) (*closed squares*), and HIVIg (*open triangles*) to gp120 by ELISA. Ig preparations were diluted 3-fold (1–0.005 mg/ml) and incubated with gp120 BaL immobilized on ELISA plate. Each data point represents the mean of optical density of duplicate wells ± S.E. A representative result of one of three independent experiments is shown. *B*, immunoblot analyses of binding of native and heme-exposed IVIg to gp120 (92RW020; BaL and JRCSF). IVIg at 10 μM was treated with 10 μM hematin. The nitrocellulose membrane with immobilized gp120 was incubated with 0.05 μM native or heme-exposed IVIg. *C*, real-time interaction profiles generated by SPR analyses of binding of native and heme exposed IVIg. IVIg at 10 μM (1.5 mg/ml) was first exposed to increased concentrations of heme (0, 1, 2, 5, 10, and 20 μM) and then injected at a final concentration of 2 μM (0.3 mg/ml) over gp120 covalently immobilized on sensor chip. *RU*, resonance units. *D*, heme concentration dependence of reactivity of IVIg to distinct variants of gp120. IVIg (20 μM or 3 mg/ml) was exposed to increasing concentrations of heme (0–64 μM) and incubated at 1 μM (0.15 mg/ml) with different variants of gp120 (92RW020, JRCSF, BaL, and CN54 immobilized on ELISA plates). Each data point represents mean optical density and ± S.E. from duplicate wells. Representative results of one of two independent experiments are shown.

Size-exclusion Chromatography

Molecular composition of native and heme-exposed pooled IgG was studied by using an FPLC ÄKTA purifier (GE Healthcare), equipped with a Superose 12 10/300 column. Pooled IgG was diluted to 20 μM in PBS and exposed to 4, 32, and 64 μM of heme. The chromatograms were recorded by using a wavelength of 280 nm.

Screening of Recombinant IgG1 for Gain of Reactivity to gp120 and Repertoire Analyses

Viral antigens from 20 μg/ml solutions in PBS (pH 7.4) were immobilized on nitrocellulose membranes using a Miniblot apparatus (Immunetics, Boston, MA). Two channels were loaded for each antigen and incubated at 4 °C overnight. Membranes were removed from the Miniblot apparatus, blocked with TBS-Tween 0.1%, and mounted perpendicularly in the Miniblot apparatus. Antibodies were treated at a 40 μg/ml concentration in PBS, either with hematin solubilized in 0.05 N NaOH (final heme concentration 20 μM) or with vehicle only (0.05 N NaOH). After a 30-min incubation, hematin-treated and native antibodies were diluted 2-fold in TBS containing 0.1% Tween 20 (TBS-T) and loaded on Miniblot channels. Likewise, a 1.5 mg/ml (~10 μM) IVIg solution in PBS was treated either with hematin (final concentration, 20 μM) or with vehicle only (0.05 N NaOH), diluted 200 times in TBS-T, and then loaded on the Miniblot system. After a 1-h incubation at room temperature, nitrocellulose membranes were removed from the Miniblot apparatus, washed for 1 h with TBS-Tween 0.1%, and incubated with horseradish peroxidase-conjugated anti-human IgG antibody (Southern Biotech). Enzymatic reaction was performed using the Pierce ECL Western blotting substrate (Thermo Scientific), and chemiluminescence was developed by 0.5-, 1-, and 4-min exposure of multipurpose film (GE Healthcare, Little Chalfont, UK).

Spot intensities at the intersections of antigens and antibodies-loaded channels were evaluated by densitometry using the Chemi-Capt/Bio-1D software (Vilber Lourmat, Torcy, France) after subtracting background intensities. Values were normalized to non-saturating IVIg intensity to take into account variations in film exposure times. Exposure time to be included in the analysis was chosen as the one giving the highest signal without saturation, for each antibody. Results are expressed as relative units. Values of the binding intensity to gp120 of each antibody before and after heme exposure were plotted. In each of these plots, a threshold that distinguishes heme-sensitive from non-sensitive antibodies was defined. The threshold was defined as the average index of binding plus three standard deviations obtained for binding of all native antibodies. Antibodies that acquire binding activity to gp120 above these thresholds after heme exposure were defined as sensitive.

RESULTS

The Presence of Heme-induced HIV-1-specific IgG Antibodies in Human Immune Repertoires—First, we tested whether seronegative human repertoires contain Abs that could gain binding to HIV-1 gp120 upon exposure to heme. As a source of IgG from humans, we used pooled human IgG obtained from >1000 different healthy blood donors. Abs from the native IgG preparation bound only negligibly to immobilized gp120 from HIV-1 BaL (clade B) (Fig. 1*A*). However, exposure of immunoglobulins to heme resulted in acquisition of a strong binding potential for gp120 (Fig. 1*A*). The binding to gp120 of heme-

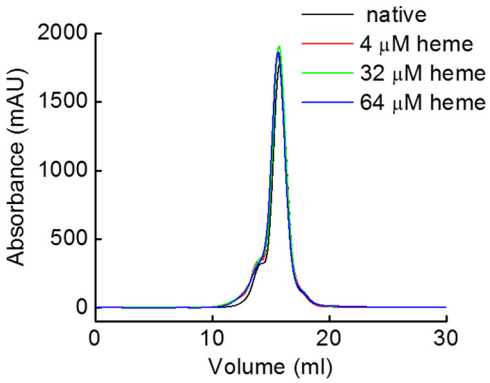

FIGURE 2. **Molecular profiles of human IgG after heme exposure.** Elution profiles obtained by size-exclusion chromatography (Superose 12 10/300 column) after injection of pooled IgG (20 μM), native or exposed to increasing concentrations of heme (4, 32, and 64 μM) are shown. *mAU*, milliabsorbance units.

exposed pooled IgG was, however, less pronounced than the binding of HIVIg, a pooled IgG preparation obtained from HIV-1 seropositive patients. The acquisition of gp120 binding specificity of IgG was further confirmed by immunoblot and SPR analyses (Fig. 1, *B* and *C*). In addition to binding to gp120 belonging to gp120 BaL, the exposure of pooled human IgG to heme uncovered binding specificities to divergent variants of gp120 as well: JRCSF (clade B), 92RW020 (clade A), and CN54 (clade C) (Fig. 1*D*).

To rule out the possibility that the appearance of binding specificity to gp120 is caused by heme-induced aggregation or other changes in molecular integrity of IgG, we studied the molecular composition of pooled immunoglobulins after exposure to heme. Exposure of IgG to heme concentrations that were well above those required for induction of binding to gp120 did not result in significant change in the molecular composition of the immunoglobulin preparation, as evident by size-exclusion chromatography (Fig. 2). These results rule out heme-induced aggregation of IgG and nonspecific increase in IgG valency as putative contributors to the induced binding to gp120.

In summary, our results demonstrate that normal human immunoglobulin repertoires contain Abs that gain binding potential to divergent strains of HIV-1 gp120 after exposure to the redox-active cofactor heme.

The Prevalence of Heme-induced HIV-1-specific Immunoglobulins in Human Immune Repertoires—To determine the prevalence of Abs that gain binding specificity to HIV-1 gp120 after heme exposure, and to decipher the molecular correlates responsible for the acquisition of HIV-1 gp120 binding, we used a repertoire of 97 human recombinant monoclonal immunoglobulins, cloned from naive and memory B cells, as well as plasma cells from seronegative individuals. All variable regions were expressed on an IgG1 framework, and therefore, all Abs have identical constant regions. This allowed us to examine the contribution of the nature of the variable regions for acquisition of gp120 binding specificity.

A Miniblot system was used to assess the reactivity of monoclonal Abs toward three divergent variants of HIV-1 gp120: 92RW020, BaL, and JRCSF. The binding of each monoclonal Ab was compared before and after exposure to heme. A representative result is depicted on Fig. 3*A*. It shows that out of nine antibodies tested, only Ab69, Ab72, and Ab76 gain binding potential for gp120 after exposure to heme. Immunoblot data were quantified using densitometry analyses (Fig. 3*B*). Altogether, 23 of the 97 monoclonal Abs (23.7%) gained significant binding activity to gp120 after heme exposure (Fig. 3*C*). Details about gene characteristics, sequence of complementarity determining regions 3 (CDR3) regions, and mutational status of variable domains of heavy and light Ig chains of the heme-sensitive antibodies are presented in supplemental Table 1. Among Abs that bind significantly to gp120, certain ones gained much higher binding potential than others (Fig. 3*B* and Table 1). The variable regions of the monoclonal Abs were cloned from naive (11.4%), memory (30.7%), or plasma B cells (57.9%). Our data reveal that there was no significant difference in the distribution of frequencies of B cell subpopulations from which heme-sensitive and non-sensitive Abs were cloned (Fig. 4*A*): naive B cells, 10.1% *versus* 15.7%; memory B cells, 31.9% *versus* 26.3%; and plasma cells, 58.0% *versus* 58.0%, respectively. The original isotype of the B cell receptor also did not correlate with the sensitivity to induction of gp120 reactivity of the recombinant Abs (Fig. 4*B*).

Most of the heme-sensitive Abs (18/23) acquired binding potential to at least two distinct gp120 variants included in the study (Fig. 3, *A* and *B*, summarized in Table 1). Previous studies have demonstrated that exposure to heme of some monoclonal Abs results in binding to unrelated antigens *i.e.* Abs acquire polyreactivity (20, 23). To determine whether Abs that gain specificity to gp120 recognize unrelated proteins, we screened the repertoire of monoclonal Abs for reactivity to different protein antigens, transferrin, tubulin, factor IX, and hemoglobin, as well as to pnC. The obtained results demonstrated that most of the monoclonal Abs in the repertoire did not significantly change their antigen binding specificity after contact with heme. However, a fraction of Abs gained a significant reactivity to different protein antigens (Fig. 5*A*). Interestingly, the exposure to heme did not uncover any Ab reactivity to carbohydrate antigen. Next, we studied whether the Ab reactivity to gp120 induced by heme correlates with acquisition of binding to unrelated proteins. The analyses of data from Fig. 5*A* revealed that 16 out of 23 heme-sensitive gp120 binding Abs, also acquired reactivity to at least single unrelated protein (summarized in Table 1). Some of heme-induced Abs, however, demonstrated exquisite specificity for gp120. For instance, although heme-exposed Ab47 has the highest binding intensity to the three gp120 variants, this Ab did not bind to any other protein or pnC (Table 1 and Fig. 5*B*). When Ab47 was excluded from correlation analyses, there was a significant correlation between the intensity of binding of heme-sensitive Abs to gp120 with intensity of binding to unrelated proteins (Fig. 5*B*). Taken together, these data suggest that most of the heme-induced gp120 binding Abs are polyreactive and that with some exceptions, the binding intensity for recognition of gp120 correlates with binding intensity for binding of unrelated proteins.

Finally, we investigated whether the intrinsic polyreactivity of native Abs is a predisposing factor for gain of binding to gp120 after interaction with heme. By using immunoblot analyses, we demonstrated that 8 out of 23 heme-sensitive Abs dis-

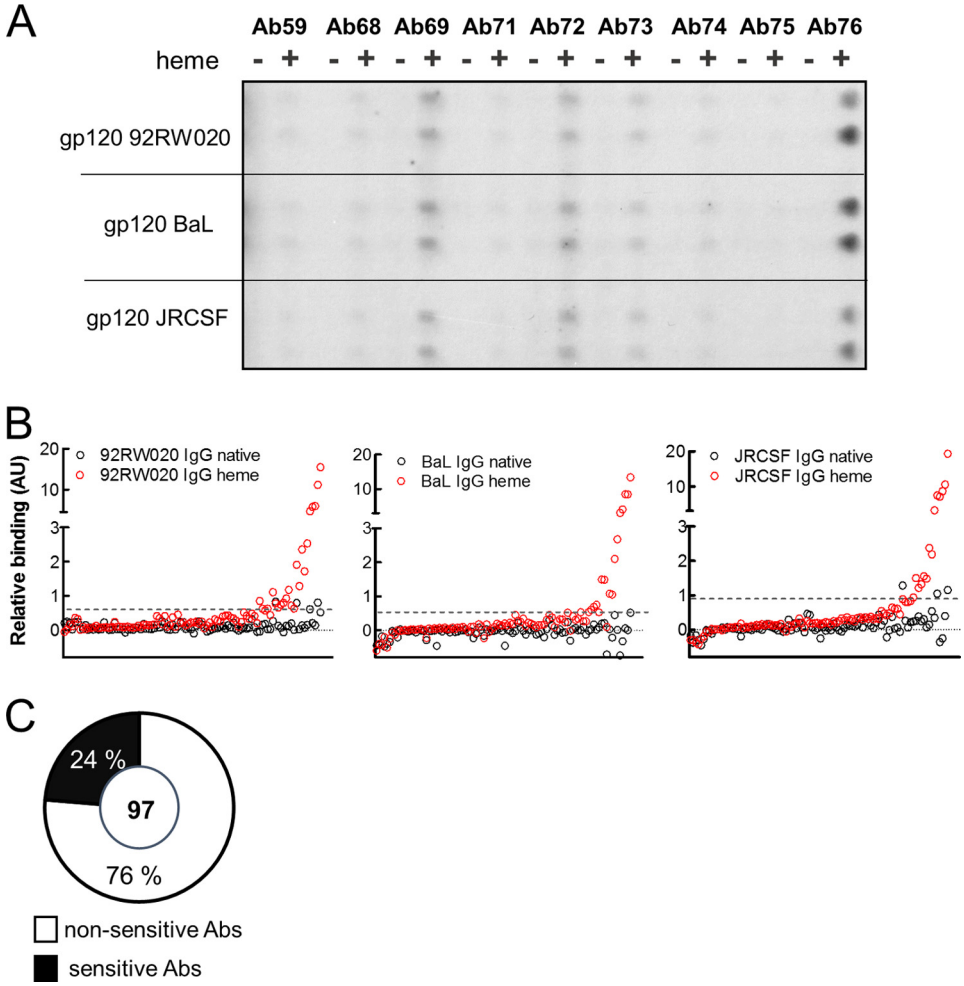

FIGURE 3. **Identification of Abs in human immunoglobulin repertoire that acquire binding activity to gp120 after exposure to heme.** *A*, representative result from immunoblot analysis of interaction of native and heme-exposed human monoclonal Abs with immobilized gp120 (variants: 92RW020, BaL, and JRCSF). Each monoclonal Ab (native and heme-exposed form) was diluted to 20 μg/ml and incubated with gp120 immobilized on nitrocellulose membrane by using the Miniblot system (Immunetics). *B*, quantitative analyses of binding of human monoclonal IgG1 antibodies to gp120. The plots represent the binding intensity to gp120 from a repertoire of 97 monoclonal IgG1 antibodies. The reactivity of each Ab before (*black circles*) and after heme treatment (*red circles*) is presented as separate points. The values were obtained by densitometric analyses of immunoblot results and after subtraction of background binding. The *dashed line* represents the average binding intensity of all native Abs plus three standard deviations. This line is used to define the threshold of sensitivity to heme. All Abs above the threshold line are able to bind significantly to gp120 and are defined as heme-sensitive. *AU*, arbitrary units. *C*, distribution of the percentage of Abs that gain binding to gp120 after heme exposure (*full area*) and those that do no change their reactivity (*empty area*).

play polyreactivity in their native state (data not shown) (Table 1). This result implies that natural polyreactivity of Abs is not a determining factor for the potential of Abs to acquire recognition of gp120 and other antigens after interaction with heme.

Molecular Correlates of Heme-induced gp120 Reactivity—The Abs used in the present work were cloned from naive, memory, and plasma B cells isolated from the synovium of three patients with rheumatoid arthritis (29). To understand the molecular correlates of cofactor-induced Ab specificity to gp120, we compared the features of the variable regions of the monoclonal Abs that acquire specificity to gp120 (heme-sensitive) with those that did not (non-sensitive) (Fig. 6). A comparison of the V-gene repertoire of the 97 human monoclonal Abs indicated an unbiased usage of Ig variable region genes, which is typical of a normal human immune repertoire (Fig. 6). The frequency of usage of V_H gene families did not differ between the two categories of Abs (Fig. 6*A*). The observed distribution, with a predominance of V_H3 family, followed a trend similar to the distribution of V_H gene families found in the IMGT human Ig genes database. Similarly, no difference in the frequency of usage of J_H families was observed (Fig. 6*A*).

The frequencies of somatic mutations in V_H regions of non-sensitive Abs showed a Gaussian distribution, typical for immune repertoires (Fig. 6*A*). In contrast, heme-sensitive Abs had a non-symmetrical distribution of the frequencies of somatic mutations in their V_H regions (Fig. 6*A*). Thus, highly mutated V_H regions (with >24 somatic mutations) were significantly less prevalent ($p = 0.048$, Fisher's exact test) among heme-sensitive as compared with non-sensitive Abs.

The complementarity-determining region of the heavy immunoglobulin chain 3 bears the highest sequence diversity among different CDR loops and plays a central role for recognition of antigen by Abs (30). The distribution of the lengths of CDR H3 regions of heme-sensitive and non-sensitive Abs did not demonstrate significant difference ($p = 0.143$, Fisher's exact test) (Fig. 6*A*). Further, the physicochemical characteris-

TABLE 1

Antigen binding characteristics of heme-induced gp120 reactive Abs

The table summarizes the reactivity of heme-treated Abs to different clades of gp120 and to unrelated proteins: transferrin, factor IX, tubulin, and hemoglobin, as well as to bacterial polysaccharide (pnC). The presented Abs gain significant reactivity to at least one of the gp120 variants. The Abs that gain significant reactivity to gp120 (as evaluated in Fig. 2) and to unrelated proteins and pnC (as evaluated in Fig. 3) upon exposure to heme are indicated by a single plus sign. Double plus signs denote reactivity of Abs higher than the positive control (heme-exposed pooled human IgG). The minus signs indicate the absence of significant binding. Natural polyreactivity of Abs refers to the reactivity of Abs in the absence of heme. This reactivity was evaluated by immunoblot analyses by measuring the recognition of bacterial proteins in lysates of *Bacillus sp.* or human liver tissue (data not shown). NA, not applicable.

	Heme-induced reactivity								
	gp120 92RW020	gp120 BaL	gp120 JRCSF	Transferrin	Tubulin	Factor IX	Hemoglobin	pnC	Natural polyreactivity
Ab12	+	−	−	NA	NA	NA	NA	NA	−
Ab13	++	+	++	++	++	++	++	−	−
Ab21	++	++	++	++	++	++	++	−	−
Ab37	+	−	−	−	−	−	−	−	−
Ab38	+	+	+	+	−	−	+	−	−
Ab47	++	++	++	−	−	−	−	−	−
Ab48	+	+	+	−	−	−	−	−	+
Ab57	+	−	−	+	+	+	+	−	−
Ab65	+	+	++	++	++	++	++	−	+
Ab67	+	+	+	+	−	+	+	−	−
Ab69	+	+	−	+	+	+	+	−	+
Ab72	−	+	−	+	+	+	+	−	+
Ab76	+	+	+	−	−	−	−	−	−
Ab99	+	−	−	+	+	+	+	−	−
Ab100	++	++	++	++	++	++	+	−	+
Ab101	+	−	+	−	−	−	−	−	−
Ab102	+	+	+	+	−	+	+	−	−
Ab104	+	+	+	+	+	+	+	−	+
Ab110	+	+	+	+	+	+	+	−	+
Ab112	+	+	−	−	−	−	−	−	+
Ab130	+	+	−	+	+	+	+	−	−
Ab131	+	+	+	−	+	−	−	−	−
Ab140	+	+	+	+	+	+	+	−	−

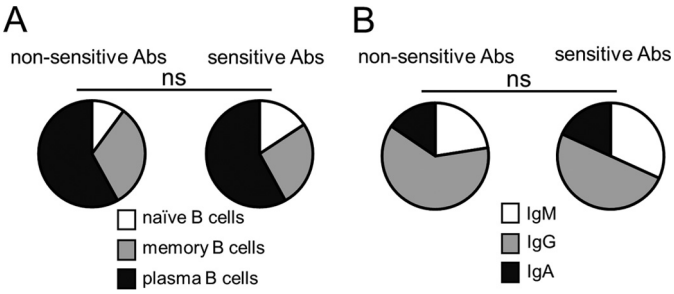

FIGURE 4. **Origin of heme-sensitive immunoglobulins.** *A*, distribution of percentages of B cell subpopulations from which Abs that were non-sensitive (*ns*, *left pie chart*) or sensitive to heme (*right pie chart*) were cloned. Differences in the percentages in the B cell subpopulations do not have statistical significance. *B*, distribution of percentages of original isotype of B cell receptors expressed on cell subpopulations from which Abs that were non-sensitive (*ns*, *left pie chart*) or sensitive to heme (*right pie chart*) were cloned. Differences in the percentages in the Ig isotypes do not have statistical significance, as indicated by Fisher's exact test.

tics of the CDR H3 regions from both groups of Abs were compared. The distribution of the number of positive, negative, or polar amino acids did not differ significantly between the two types of Abs (Fig. 6*A*). However, there was a strong tendency for a higher number of negative ($p = 0.086$, Fisher's exact test) and polar ($p = 0.092$, Fisher's exact test) amino acid residues in CDR H3 regions of heme-sensitive Abs (Fig. 5*A*). The overall hydropathy index (GRAVY (grand average of hydropathicity)) of the CDR H3 showed identical distribution between the two groups of Abs (data not shown).

The majority of cloned Abs used κ light chains, in both the sensitive and the non-sensitive groups of Abs (Fig. 6*B*). Sequence analyses of the light chain variable region genes revealed that there was a similar distribution between heme-sensitive and non-sensitive Abs in the number of somatic mutations, size of CDR L3, or physicochemical properties of CDR L3 (Fig. 6*B*).

DISCUSSION

In the present study, we demonstrate that seronegative immunoglobulin repertoires contain Abs that acquire the potential to recognize gp120 from HIV-1 after exposure to heme. We estimated the prevalence of these Abs in human immune repertoires and elucidated the molecular features of their variable regions associated with sensitivity to the cofactor molecule. Using a panel of 97 monoclonal Abs cloned from B cells from seronegative individuals, we found that ~24% of Abs acquire binding specificity to gp120 after exposure to heme. Most of the Abs (~70%) that acquired reactivity to gp120 also become polyreactive following heme exposure. No difference in the sensitivity to heme was seen whether Abs were originally expressed by naive, memory, or plasma cells. Because all of the studied Abs possess an identical heavy chain constant region (γ1 H-chain) and the majority is associated with a κ light chain, these results imply that the sensitivity of immunoglobulins to heme is a property determined by the sequence of the variable region genes. Most of the heme-exposed Abs acquire reactivity to three divergent variants of gp120, indicating that cofactor exposure gives these Abs the ability to adapt to the enormous sequence heterogeneity of the antigen.

Previous studies have demonstrated that exposure of some monoclonal Abs to the redox cofactor heme results in acquisition of reactivity to different structurally unrelated antigens (20, 31). Spectroscopic analyses revealed that this phenomenon is mediated by binding of the macrocyclic cofactor molecule to the variable region of immunoglobulins (7, 31). The heme molecule may provide versatile types of non-covalent interactions:

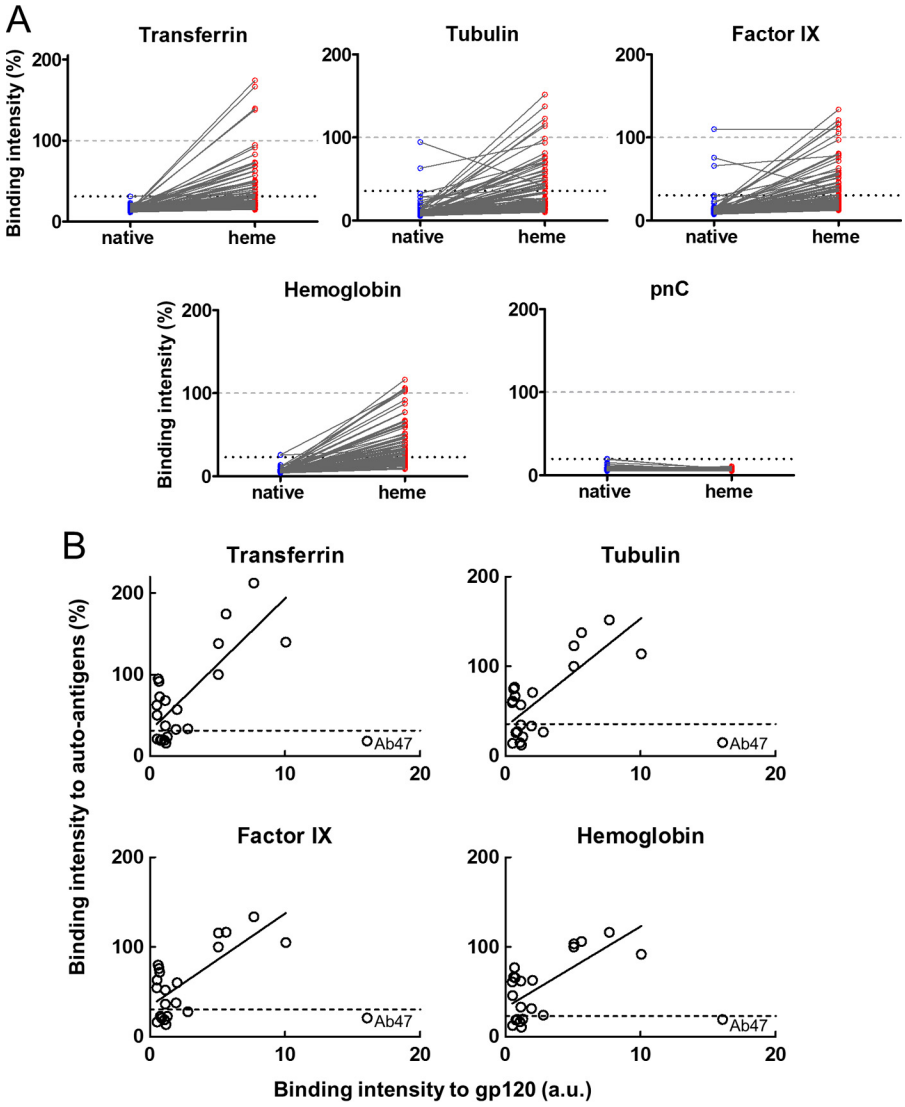

FIGURE 5. **Heme-mediated polyreactivity of monoclonal Abs.** *A*, quantitative analyses of reactivity of the repertoire of human monoclonal IgG1 Abs to human transferrin, porcine tubulin, human factor IX, human hemoglobin (apo form), and pneumococcal C-polysaccharide. The plots represent the binding intensity of each Ab in the absence (*blue circles*) and presence of heme (*red circles*), obtained by ELISA assay. Native and heme-exposed Abs were incubated at concentration of 6.25 μg/ml with immobilized antigens. The *black dotted lines* represent the mean binding intensity of native Abs plus five standard deviations. These values were used as thresholds to identify the Abs that acquire significant binding to antigen upon heme exposure. The binding intensity of each Ab was presented as a percentage of the binding intensity of the positive control (50 μg/ml of heme-exposed pooled human IgG). The *dashed gray lines* represent the reactivity of positive control, which is considered as 100%. *B*, correlation of Ab reactivity to gp120 with reactivity to unrelated proteins. The correlation analyses were performed by using Abs that acquire significant reactivity to gp120 after heme exposure (identified in Fig. 3), excluding Ab47. Shown is correlation of Abs binding to gp120 with binding to: factor IX (*FIX*), $p = 0.0001$, $R^2 = 0.54$; transferrin, $p < 0.0001$, $R^2 = 0.61$; tubulin, $p < 0.0001$, $R^2 = 0.58$; and hemoglobin, $p = 0.0004$, $R^2 = 0.487$. The *black dashed line* defines the threshold of positivity for reactivity to proteins other than gp120. The linear regression plot was generated by excluding the value for the reactivity of Ab47, which is indicated on the graphs. *a. u.*, arbitrary units.

hydrogen bonds, van der Waals, π-stacking, ionic, and metal coordination interactions. Such a property could explain the tendency of heme to interact with many different proteins (32, 33). The variable regions of Abs are characterized with an enormous sequence diversity that is predominantly concentrated in the CDR (30). Thus, a fraction of immunoglobulins in healthy immune repertoires may carry sequence motives that are appropriate for binding of heme or other cofactors (1, 2, 14, 17). However, the molecular characteristics of the variable regions of these Abs had not been systematically elucidated. In the current study, we sought to understand the features of the variable regions that are responsible for acquisition of novel antigen binding specificity upon exposure to cofactor. To this end, we used a repertoire of human immune Abs. No bias in V_H and V_L gene family usage or difference in the lengths of CDR H3 and CDR L3 regions were observed for Abs that acquire promiscuous reactivity to gp120. Our analyses demonstrated similarities in the frequencies of positive, negative, or polar amino acid residues as well as in the overall hydrophobicity of CDR H3 between cofactor-sensitive and non-sensitive Abs. However, Abs in the repertoire that acquire binding specificity to gp120 after interaction with heme possess significantly less mutated variable regions as compared with Abs that do not change their antigen binding specificity. The immunoglobulins with a lower number of somatic mutations are characterized with more pliable antigen-binding sites (34–41). Presumably, a lower number of

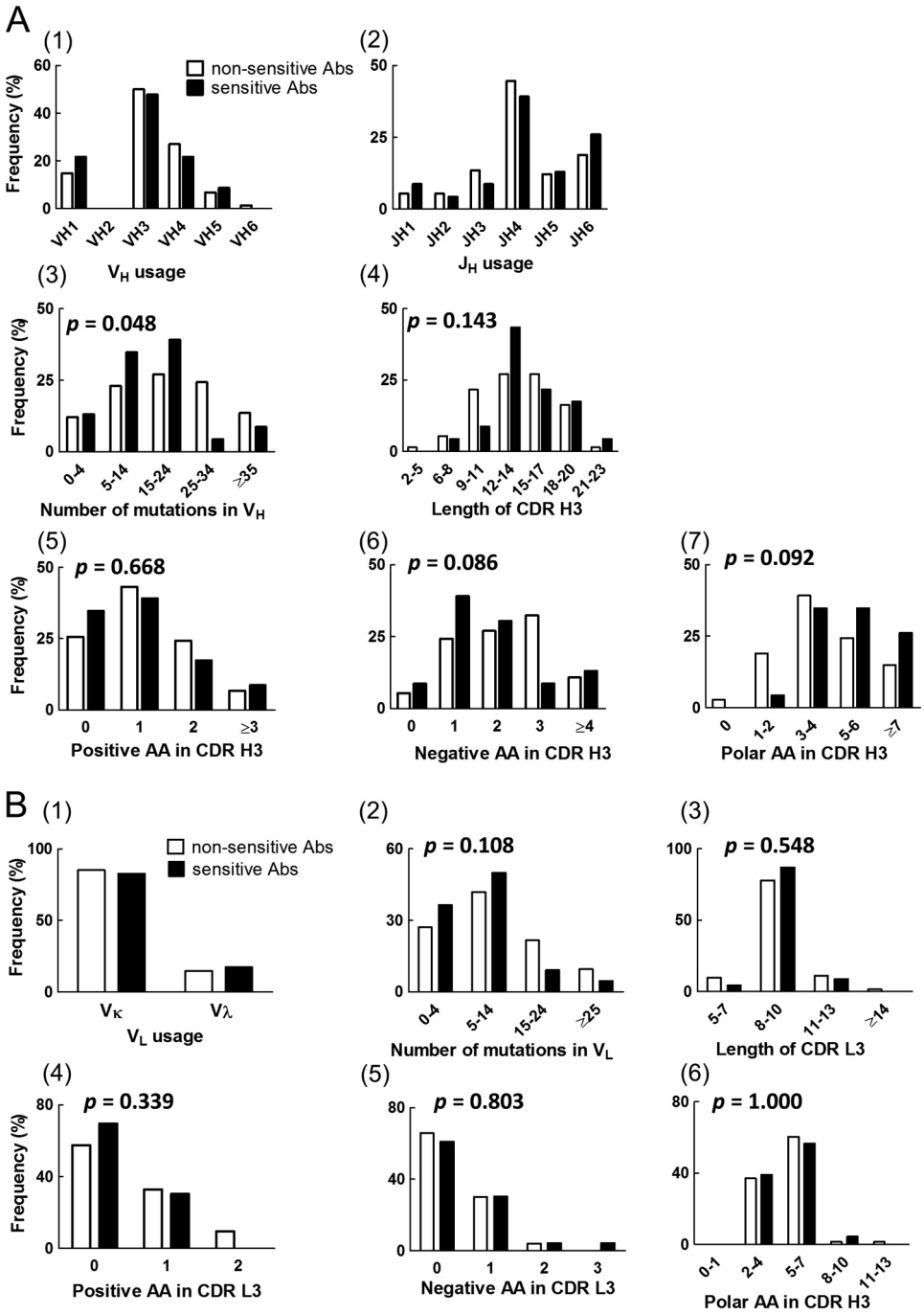

FIGURE 6. **Analyses of molecular features of variable regions of heavy and light Ig chains of heme-induced gp120 reactive monoclonal Abs.** *A*, frequency distribution analyses of: gene families encoding heavy chain variable regions (*panel 1*); gene families encoding J_H fragments (*panel 2*); number of somatic mutations in V_H regions (*panel 3*); length of CDR H3 loop (*panel 4*); number of positive amino acid residues (*AA*) in CDR (*panel 5*); number of negative amino acid residues in CDR H3 (*panel 6*); and number of polar amino acid residues in CDR H3 (*panel 7*). The w*hite bars* represent the distribution of V_H features of antibodies that did not acquire binding specificities after heme exposure. The *black bars* represent the frequency distribution of V_H characteristics of heme-sensitive Abs. Statistical analyses of frequency distributions by Fisher's exact test indicated significance between heme-sensitive and non-sensitive Abs in the case of number of somatic mutations ($p = 0.048$, threshold value, 24). The frequency distributions of other characteristics of variable regions did not show statistical significance ($p > 0.05$, Fisher's exact test). *B*, analyses of molecular features of variable regions of light Ig (V_L) chains of heme-induced gp120 binding monoclonal Abs. Shown are frequency distributions of: isotype of light Ig chain (*panel 1*); number of somatic mutations in the variable regions of light Ig chains (*panel 2*); length of CDR L3 (number of amino acid residues) (*panel 3*); number of positive amino acid residues (*AA*) in CDR L3 (*panel 4*); number of negative amino acid residues in CDR L3 (*panel 5*); and number of polar amino acid residues in CDR L3 (*panel 6*). The analysis was performed on 97 Abs evaluated by immunoblot analyses (Fig. 2). The *white bars* depict distribution of V_L characteristics of Abs that did not acquire binding potential to gp120 after heme exposure, defined as non-sensitive Abs. The *black bars* depict the distribution of V_L characteristics of Abs that acquire binding to gp120 after heme exposure, defined as heme-sensitive Abs. Statistical analyses were performed by using Fisher's exact test.

somatic mutations in the variable region of heme-sensitive Abs will permit more extensive conformational rearrangements in these regions, increasing the probability for accommodation of the cofactor molecule to the variable region (42, 43). The absence of any typical sequence motif in variable regions of Abs that acquire binding specificity to gp120 could be explained with the high bind-

ing promiscuity of heme. Indeed, computational and experimental studies have revealed that heme-binding motifs on distinct proteins are characterized by a vast diversity of sequences and structures (44, 45). We hypothesized that heme could interact with Abs in many alternative ways, depending on the sequence of the variable region. Enormous heterogeneity of sequences of antigen-binding sites may offer different configurations appropriate for heme accommodation and for induction of novel antigen binding specificity. Indeed, our data indicated that binding intensity and the tendency for polyreactivity considerably differ between heme-sensitive Abs. This observation ruled out the possibility that heme binds to invariant sequence present in different Abs and that the binding occurs in an identical manner.

The mechanism underlying the appearance of novel antigen specificity upon binding of cofactor molecule to Abs is not yet well understood. One may envisage that heme binding to Abs induces conformational rearrangements in variable regions that result in a change of the antigen specificity. Abs with a lower number of somatic mutations would be more prone to such structural rearrangements (34–38). In addition, the unique chemistry of heme could extend the binding potential intrinsic to the polypeptide chain of Abs; thus, transient heme binding could serve as a cofactor of Abs for interaction with antigens (21). Recently, we performed a study with a prototypic heme-binding antibody that acquires reactivity to gp120 upon heme exposure, selected from the presented repertoire (31). Our data demonstrate that following interaction with heme, this Ab acquires antigen binding promiscuity and recognizes divergent variants of gp120 with quantitatively similar kinetic and thermodynamic parameters. Conversely, a broadly neutralizing Ab isolated from an HIV-1-infected patient displayed more discriminative interactions and was not able to recognize all the variants of gp120 included in the study. The results obtained in the latter study also suggest that cofactor-bound Abs use the unique chemistry of the heme molecule to recognize gp120, wherein IgG-bound heme serves as an interfacial bridge to connect Ab and gp120 (31). Importantly, heme and its analogues have been demonstrated to bind to gp120 in the V3 region (46–49). Epitope mapping predicted the same region as the most probable binding site of cofactor-bound Ab (31), thus supporting the hypothesis that the cofactor molecule serves as an interfacial bridge between the antibody and antigen. Therefore, we do not exclude the possibility that binding of heme to gp120 might result in recruitment of cofactor binding Abs in their apo-form. However, it remains to be estimated whether all cofactor-sensitive molecules in human immune repertoires utilize heme as an interfacial bridge for interacting with gp120.

Typical hemoproteins use heme as a prosthetic group for gas and electron transport and catalysis of versatile oxidative reactions (32). The absence of heme completely abrogates the functions of hemoproteins. Heme has also been demonstrated to bind to many proteins that are not conventional hemoproteins. Such an interaction promiscuity of heme plays an important role in the regulation of diverse biological processes such as gene expression, protein degradation, signal transduction, ion channel conductivity, circadian rhythms, and immune reactions (50–59). In contrast to its role in hemoproteins, the regulatory functions of heme are exerted only by transient interactions with polypeptide chains. The transient interaction of heme with a fraction of circulating Abs that could use the properties of the cofactor to extend their antigen binding repertoire may represent yet another regulatory function of heme. Thus, cofactor-binding Abs present in the circulation might be regarded as a source of antigen binding specificities in immune repertoires (60). The novel antigen specificities of these Abs would be recruited only as result of certain pathological conditions. For example, free extracellular heme is released in large quantities in the circulation in the course of different disorders such as malaria, sickle cell disease, hemolytic anemia, β-thalassemia, sepsis, and ischemia-reperfusion (25, 27, 61). It is noteworthy that HIV-1 has high sensitivity to heme, which has been explained by different mechanisms (48, 62–65). Accordingly, a recent clinical study has revealed that the incidence of HIV-1 infection in patients with sickle cell disease, who might have intravascular hemolysis and release of free heme, is significantly lower as compared with the normal population (66). Our data suggest that the fraction of cofactor-binding Abs might also contribute to the documented inhibitory effect of heme on HIV-1. Importantly, heme-bound Abs display antigen binding polyreactivity (7, 31). Our analyses revealed that a substantial fraction (16/23 or ~70%) of heme-induced gp120 binding Abs is polyreactive and could interact with unrelated protein antigens. Different studies have highlighted antigen binding polyreactivity as one of the hallmarks of neutralizing Abs generated during HIV-1 infection (67–72). In addition, the potential of some HIV-1-neutralizing Abs to recognize different structures was proposed to contribute directly to neutralization of the virus (69). Therefore, it would be important to understand whether heme-bound polyreactive Abs could also be efficient for neutralization of HIV-1.

In summary, we provide here evidence that human immune repertoires contain a significant fraction of Abs with cofactor-induced antigen binding specificity to HIV-1 gp120. These Abs possess fewer mutated variable regions than Abs that do not acquire new antigen binding specificity. Further work is required, however, to delineate the significance of Abs with cofactor-induced gp120 specificity in immune responses, to understand the molecular mechanisms of binding to gp120, and to exploit their therapeutic potential.

Acknowledgment—We thank the NIH AIDS Reagent Program, Division of AIDS, NIAID, at the National Institutes of Health for providing us vital materials.

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